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International Multicenter Evaluation of the DiversiLab Bacterial Typing System for *Escherichia coli* and *Klebsiella* spp.

Guido M. Voets,[a] Maurine A. Leverstein-van Hall,[a,b] Susanne Kolbe-Busch,[c] Adri van der Zanden,[d] Deirdre Church,[e] Martin Kaase,[f] Andrea Grisold,[g] Mathew Upton,[h]* Elaine Cloutman-Green,[i] Rafael Cantón,[j] Alexander W. Friedrich,[k] Ad C. Fluit,[a] on behalf of the DiversiLab Study Group

Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, the Netherlands[a]; Bronovo Hospital, the Hague and Diaconessenhuis, Leiden, the Netherlands[b]; Institut für Medizinische Mikrobiologie und Krankenhaushygiene, Düsseldorf, Germany[c]; Laboratorium Microbiologie Twente Achterhoek, Enschede, the Netherlands[d]; Department of Pathology & Laboratory Medicine and Medicine, Calgary, Canada[e]; Department of Medical Microbiology, Bochum, Germany[f]; Institute of Hygiene, Microbiology and Environmental Medicine, Graz, Austria[g]; University of Manchester School of Medicine, Manchester, United Kingdom[h]; Great Ormond Street Hospital for Children, London, United Kingdom[i]; Servicio de Microbiología, Hospital Universitario Ramón y Cajal and Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain[j]; Department of Medical Microbiology, University of Groningen, Groningen, the Netherlands[k]

Successful multidrug-resistant clones are increasing in prevalence globally, which makes the ability to identify these clones urgent. However, adequate, easy-to-perform, and reproducible typing methods are lacking. We investigated whether DiversiLab (DL), an automated repetitive-sequence-based PCR bacterial typing system (bioMérieux), is suitable for comparing isolates analyzed at different geographic centers. A total of 39 *Escherichia coli* and 39 *Klebsiella* species isolates previously typed by the coordinating center were analyzed. Pulsed-field gel electrophoresis (PFGE) confirmed the presence of one cluster of 6 isolates, three clusters of 3 isolates, and three clusters of 2 isolates for each set of isolates. DL analysis was performed in 11 centers in six different countries using the same protocol. The DL profiles of 425 *E. coli* and 422 *Klebsiella* spp. were obtained. The DL system showed a lower discriminatory power for *E. coli* than did PFGE. The local DL data showed a low concordance, as indicated by the adjusted Rand and Wallace coefficients (0.132 to 0.740 and 0.070 to 1.0 [*E. coli*] and 0.091 to 0.864 and 0.056 to 1.0 [*Klebsiella* spp.], respectively). The central analysis showed a significantly improved concordance (0.473 to 1.0 and 0.290 to 1.0 [*E. coli*] and 0.513 to 0.965 and 0.425 to 1.0 [*Klebsiella* spp.], respectively). The misclassifications of profiles for individual isolates were mainly due to inconsistent amplification, which was most likely due to variations in the quality and amounts of the isolated DNA used for amplification. Despite local variations, the DL system has the potential to indicate the occurrence of clonal outbreaks in an international setting, provided there is strict adherence to standardized, reproducible DNA isolation methods and analysis protocols, all supported by a central database for profile comparisons.

The prevalence of successful multidrug-resistant clones, e.g., *Klebsiella pneumoniae* ST258 and *Escherichia coli* ST131, is increasing globally (1–4). The spread of these high-risk clones is aided by increases in international travel, medical treatment abroad, and repatriated patients (5, 6). The ability to identify these epidemic clones is of importance for understanding the epidemiology of these isolates and may alert hospitals to the emergence of epidemic strains. This requires a reliable typing method capable of identifying the epidemic clones that can be used at different centers together with an internationally accessible database for comparisons (7). Pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) have been used for this purpose. The main drawback of PFGE, however, is poor reproducibility due to technical variations and the time-consuming nature of the method, whereas MLST lacks sufficient discriminatory power. Multiple-locus variable-number tandem-repeat analysis (MLVA) and amplified fragment length polymorphism analysis (AFLP) are also typing methods with databases, but these methods are not widespread and also have technical limitations (8–10).

The DiversiLab (DL) bacterial typing system (bioMérieux, Marcy l'Etoile, France), which allows results to be obtained within a day, may offer an alternative, although it is based on repetitive-sequence-based PCR (rep-PCR), which also shows poor reproducibility (11, 12). By standardization of the procedures (PCR and analysis of the amplification products) and the use of a commercial microfluidics system, the DL system has improved reproducibility and the potential for multicenter comparisons of typing data, thereby possibly facilitating the identification of international clones. The method can be easily introduced into routine settings and requires less hands-on time than PFGE. The ease of use is also facilitated by the associated website that allows easy analysis and visualization of the data. However, comparisons between different centers have not yet been performed. The aim of this study was to evaluate the interlaboratory reproducibility of DL analysis for *E. coli* and *Klebsiella* species isolates in an international multicenter setting. Eleven centers in six countries typed 39 *E. coli* and 39 *Klebsiella* species isolates, which were previously characterized by PFGE and represent either outbreaks or unique isolates.

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Address correspondence to Ad C. Fluit, a.c.fluit@umcutrecht.nl.
* Present address: Mathew Upton, School of Biomedical and Healthcare Sciences, University of Plymouth, Plymouth, United Kingdom.
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MATERIALS AND METHODS

Isolates and centers. In total, 39 *E. coli* and 39 *Klebsiella* species (34 *K. pneumoniae* and 5 *Klebsiella oxytoca*) isolates that had been typed previously by PFGE were selected from the collections of the Hospital Hygiene Department of the University Medical Center Utrecht and a study on the population distribution of β-lactamases conferring resistance to broad-spectrum cephalosporins in human clinical isolates in the Netherlands (13). The clustering in PFGE was based on a cutoff of 80% and was in agreement with epidemiological data. Repeated PFGE typing of the two groups confirmed one cluster of 6 isolates, three clusters of 3 isolates each, three clusters of 2 isolates, and 18 isolates with unique profiles. The isolates were initially identified by standard microbiology methods and later confirmed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Germany).

The isolates, from a single plate, were shipped on M40 Transystem Amies agar gel transport swabs (Copan Italia SpA, Brescia, Italia) to the 11 participating centers in 6 countries, including 5 countries in Europe (Austria, England, Germany, Spain, and the Netherlands) and Canada.

Typing. All centers used the same protocol. The DNA was isolated using an UltraClean microbial DNA isolation kit (Mo-Bio Laboratories Inc., Carlsbad, CA, USA) with two changes for the *Klebsiella* species isolates, according to the manufacturer's instructions. Based on previous experience (8), it was recommended that all centers use a 10-μl loop of bacteria and 900 μl of the MD3 solution. A highly sensitive spectrophotometer, the NanoDrop, or an equivalent instrument was used to quantify the DNA. The minimal required concentration was 25 ng/μl. The DNA was required to have an optical density at 260 nm (OD_{260})/OD_{280} ratio of >1.7 and an OD_{260}/OD_{230} ratio of >1.3. Each center performed PCRs with AmpliTaq (Invitrogen, Breda, the Netherlands) and the kits specified by the manufacturer for *E. coli* (kit no. 270613) and *Klebsiella* spp. (kit no. 270615). The PCR products were analyzed using standard chips (no. 270670). Each center uploaded the chip results to its own bioMérieux DiversiLab website for local analysis.

Data analysis and statistical methods. The analysis of the profiles was performed at two levels. First, all profiles were analyzed at the level of the individual laboratory, and second, all profiles were examined by the staff at the coordinating center. All centers received the same protocol for analysis of the data at the first level. The recommendation was that DL results to be used for comparisons should lack automatic warnings and have peak intensities of ≥100 for at least one peak. The analysis was performed using Pearson's correlation in the dedicated DL software of the manufacturer (version 3.4). Isolates with similarities of <95% were considered different, and isolates with similarities of >98% were considered indistinguishable. All isolates with similarities of >95% and <98% were judged manually using the pattern overlay of the analysis tool in the software.

The statistical analysis (adjusted Rand's and Wallace's coefficients) was performed using the online tool of the Instituto de Medicina Molecular of the University of Lisbon (http://darwin.phyloviz.net/ComparingPartitions/index.php). The discriminatory power was estimated by Simpson's index of diversity (14). Ninety-five percent confidence intervals (95% CIs) for discriminatory indices were calculated according to the method of Grundmann et al. (15). Nonoverlapping CIs were regarded as representing statistically significant differences in discriminatory power (15).

Isolates designated nontypeable, defined as failing to meet the minimal criteria of peak intensities, were included in the statistical analysis as unique values. All nonprocessed and nonviable isolates and isolates that could not be amplified, defined as those failing to generate an amplification product in the PCR step, were removed from the statistical analyses. PFGE data were not communicated to the local centers.

The central analysis was performed by the chief investigators (G.M.V. and A.C.F.). The data were also transferred to a dedicated website created by bioMérieux with the same DL analysis software as that used by the individual centers for the analysis of the combined data. The data were judged manually using the pattern overlay of the analysis tool in the software. The statistical analysis was performed as described above for the local analyses.

RESULTS

The typing data for 425 of 429 *E. coli* and 422 of 429 *Klebsiella* species samples were available for analysis. Three of the *E. coli* samples were not processed (<1%), and one showed no amplification in one of the centers (<1%). Similarly, seven of the *Klebsiella* samples (1%) were not processed (<1%). The analyses performed by the individual centers were confirmed by the central laboratory.

The local analyses showed little consensus (Fig. 1A), which was confirmed by the adjusted Rand and Wallace coefficients (see Tables S1 and S2 in the supplemental material). Nevertheless, the local analyses of the *E. coli* DL data agreed in 96% of the cases for half of the unique isolates according to PFGE (Fig. 1A, bottom 9 isolates). A similar level of consensus was obtained for the cluster consisting of isolates 31E and 32E and the cluster consisting of isolates 14E, 24E, and 25E. However, there was no consensus in the assignment for the isolates in the other three clusters. The lack of consensus was confirmed by the adjusted Rand and Wallace coefficients (see Tables S1 and S2 in the supplemental material), with mean values of 0.357 and 0.389, respectively. The two statistical analyses showed poor overall values (ranges, 0.132 to 0.740 and 0.070 to 1.0, respectively). An exception for the adjusted Wallace coefficients was the comparison with center 3, which was due to the fact that this analysis resulted in one big cluster that encompassed the smaller clusters identified by other centers.

For *E. coli*, the DL analyses from the local centers showed less discriminatory power than did the PFGE analyses. Examples of this are the DL clusters with isolates 31E and 32E, 18E and 37E, and 14E, 24E, and 25E (Fig. 1A).

The central analysis of the *E. coli* DL data also resulted in less discriminatory power than did the PFGE analysis, which is reflected by the lower Simpson index of diversity (see Table S3 in the supplemental material). The Simpson index of diversity was 0.964 (95% CI, 0.935 to 0.992) for PFGE and ranged from 0.709 (95% CI, 0.550 to 0.867) to 0.864 (95% CI, 0.767 to 0.961). However, it showed improved consensus between the data from the different centers (Fig. 1B; see also Tables S4 and S5 in the supplemental material) compared to that for the local analyses. This was most apparent in the cluster consisting of 22E and 39E, the cluster consisting of 18E and 37E, and the cluster consisting of 34E up to and including 11E in Fig. 1B, which improved significantly in concordance, as is reflected in the adjusted Rand and Wallace coefficients (see Tables S4 and S5 in the supplemental material). The adjusted Rand coefficients have a mean value of 0.8 (range, 0.473 to 1.0). The average was mainly lowered by the results obtained by center 11. The directional adjusted Wallace coefficient also reflected the improved concordance with a mean value of 0.83, and for 6 of the 11 centers, the mean was >0.9 (range, 0.290 to 1.0).

The *Klebsiella* spp. were not separated into different species, as most of them were *K. pneumoniae* ($n = 34$) or *K. oxytoca* ($n = 5$), and these were unique isolates based on PFGE. The assignments of *Klebsiella* spp. agreed in 97% of the cases for two-thirds of the unique isolates according to PFGE (Fig. 2A, last 12 isolates). The concordance of the isolates belonging to a cluster was larger than that found for *E. coli*, although more outliers were present. This is reflected in the overall higher and more consistent values of the

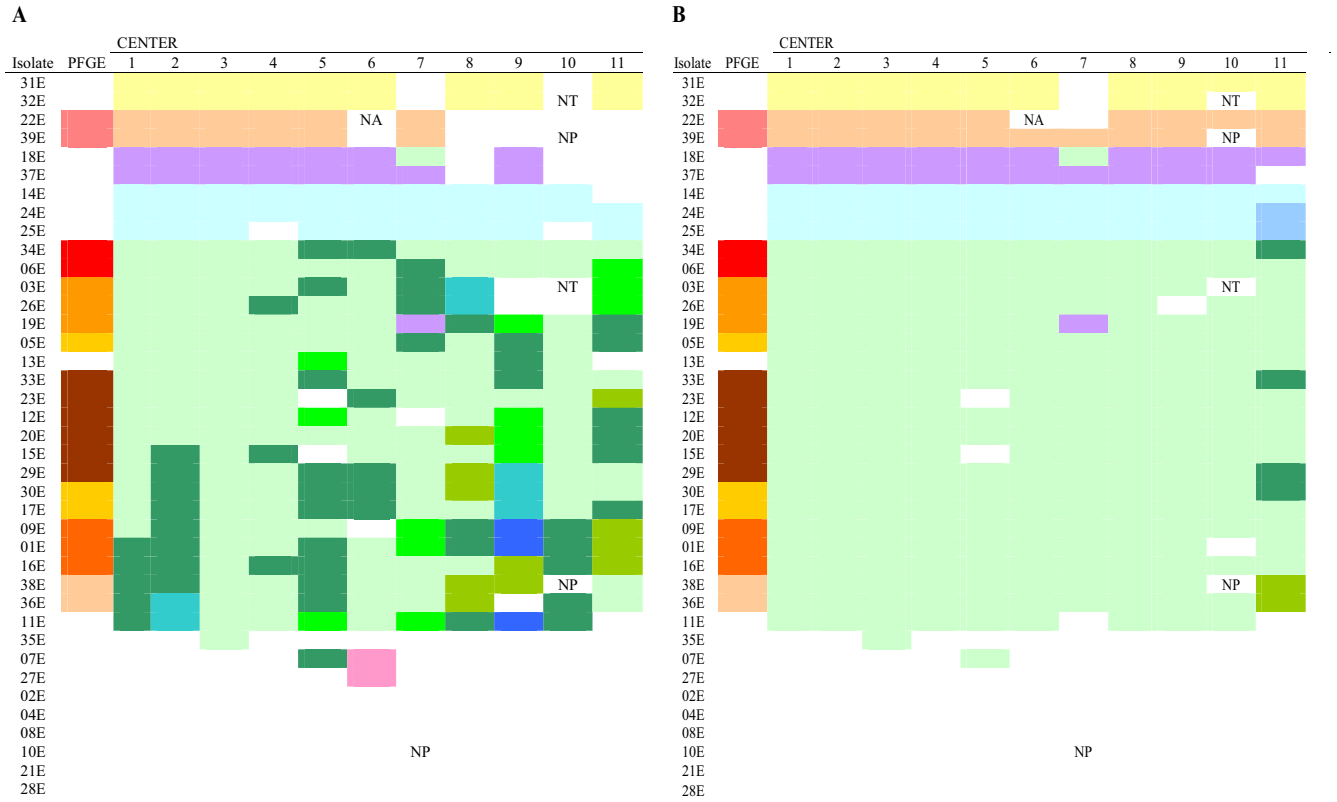

FIG 1 (A) Comparison of the local clustering of *E. coli* and the clustering of the isolates using PFGE. The isolates belonging to one cluster according to the local analysis or PFGE are indicated by the same color. (B) Comparison of the central clustering of *E. coli* and the clustering according to PFGE. The isolates belonging to one cluster according to central analysis or PFGE are indicated by the same color. The isolates left blank were considered unique isolates according to the central analysis. NT, nontypeable; NP, not processed by the center; NA, no amplification.

adjusted Rand and Wallace coefficients (see Tables S6 and S7 in the supplemental material), although both statistical analyses show poor overall values (ranges, 0.091 to 0.864 and 0.056 to 1.0, respectively). An exception to this finding for the adjusted Wallace coefficients is center 9, where the analysis identified one big cluster which encompassed the smaller clusters that the other centers identified.

In comparison to the PFGE analysis for *Klebsiella* spp., the DL analysis did not show a significant difference in the discriminatory power in the local analyses (Fig. 2A; see also Tables S6 and S7 in the supplemental material). Examples of this are the isolates 03K and 35K and 37K.

A marked improvement in the degree of concordance between the centers was obtained by the central analysis, and the discriminatory power remained acceptable (Fig. 2B; see also Table S3 in the supplemental material). Most notably, the formation of the cluster containing the isolates 23K, 09K, and 36K, the cluster consisting of 02K and 33K, the cluster consisting of 26K and 31K, the cluster consisting of 37K and 35K, and the cluster consisting of 21K and 38K improved significantly in concordance. This is reflected in the statistical analysis (see Tables S8 and S9 in the supplemental material); the adjusted Rand coefficients have a mean value of 0.71 (range, 0.513 to 0.965). The values of the adjusted Wallace coefficient also increased in comparison to that for the local analyses to a mean value of 0.73 (range, 0.425 to 1.0). We noted that although different clusters can be assigned to the isolates 02K, 05K, 06K, 11K, 26K, 28K, 31K, and 33K, the patterns of these clusters were closely related, and these clusters and unique isolates may be considered a clonal complex (Fig. 3).

DISCUSSION

We performed an international multicenter evaluation of the DiversiLab bacterial typing system for *E. coli* and *Klebsiella* spp. in order to assess whether the DL system is suitable for comparing isolates analyzed at different centers. Some local studies (8, 16–18) showed that the DL system performed well for several species, e.g., *Klebsiella* spp., and to a lesser extent for some others, e.g., *E. coli*. Moreover, it was shown to identify some of the circulating high-risk clones such as *E. coli* ST131 harboring CTX-M-15 extended-spectrum β-lactamase and clonal complex 147 from *K. pneumoniae* expressing carbapenemases (19, 20).

In our study, 11 centers from six different countries typed 39 *E. coli* and 39 *Klebsiella* species isolates that were selected based on PFGE results. The main findings of the study were that (i) the DL system had a lower discriminatory power for the *E. coli* isolates than did PFGE, (ii) the clustering obtained by the different centers was only partly concordant, and (iii) the central analysis improved the clustering to an acceptable level. In comparison to PFGE typing, the DL system had less discriminatory power, creating larger clusters and clustering of isolates that are considered unique by using PFGE (Fig. 1 and 2). This has also been demonstrated by other studies (21–23). The isolates considered to be different by the DL system are also considered to be different by the PFGE.

A number of factors might have contributed to the less-than-

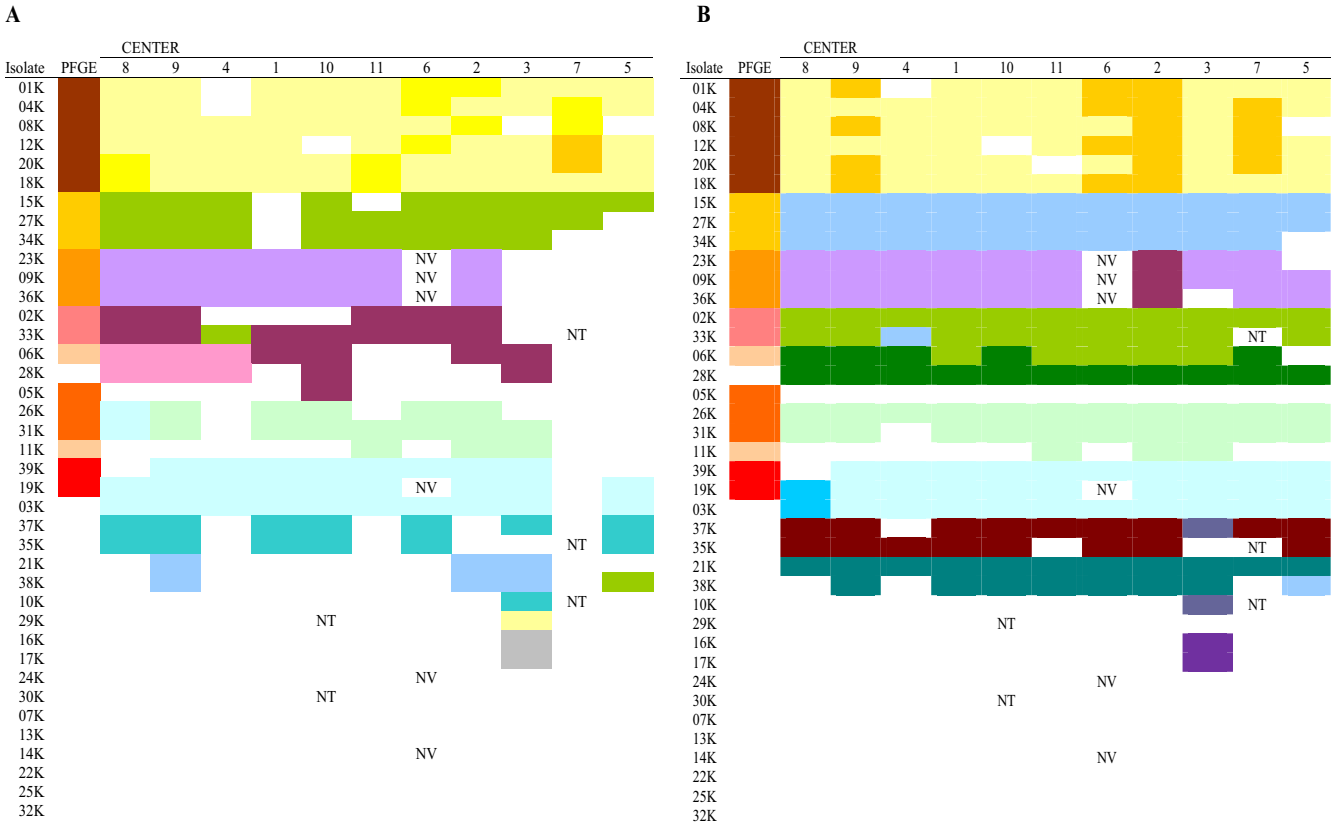

FIG 2 (A) Comparison of the local clustering of *Klebsiella* spp. and the clustering of the isolates using PFGE. The isolates belonging to one cluster according to the local analysis or PFGE are indicated by the same color. (B) Comparison of the central clustering of *Klebsiella* spp. and the clustering according to PFGE. The isolates belonging to one cluster according to the central analysis or PFGE are indicated by the same color. The isolates left blank were considered unique isolates according to the central analysis. NT, nontypeable; NV, nonviable.

optimal concordance between the centers. The main factors are incorrect clustering by Pearson's correlations and the misclassification of isolates due to variations in the amplification products between isolates. In some cases, the amplification signals were faint or even completely absent, causing incorrect clustering by Pearson's correlations. In the central analysis, which relied completely on the interpretation of the overlays, more consistent clustering was obtained (Fig. 1B and 2B). This was further aided by the fact that, for each isolate, at least 10 replicates were available. This allowed the assessment and mitigation of continuous minor changes in the patterns of the isolates when the individual clusters were examined. Sometimes the replicates for the same isolates did not cluster next to each other but across the whole cluster (Fig. 3). These data indicate that the reproducibility of one or more of the steps before analysis is insufficient. Several issues that might be responsible for this lack of reproducibility are (i) variations between the persons performing the assays, (ii) inconsistent amplification, and (iii) inconsistent quality and/or amounts of the DNA obtained during its isolation. However, this also indicates the importance of a central database for comparing isolates. Moreover, person-to-person variations, variations in thermocycler performance, and variations in the quality and amounts of the DNA isolated are known from the experiences at the central laboratory to lead to variations in the results (A. C. Fluit, unpublished data). This study lacked an experimental design to measure person-to-person variations within a center. Regardless, it is difficult to control for person-to-person variations, particularly within a multicenter setting.

Inconsistent DNA amplification is most likely not due to the PCR kits used, as these are quality checked for consistent performance, and the same batches were used in all centers included in this study. However, the thermocycler used may be a source of variation, especially since during the amplification process in a rep-PCR-based system, sometimes less-than-optimal interactions between the primers and target occur, and slight differences in the initial conditions, such as small variations in the primer concentrations, can have a major impact on the results. Although it is possible at a local level to assign a single machine to the DL assays, this is not feasible in a multicenter setting. One of the most important parameters in amplification is the quality and amount of DNA added. The study protocol required a minimal amount and quality of the DNA (25 ng/μl and an OD_{260}/OD_{280} ratio of >1.7 and an OD_{260}/OD_{230} ratio of >1.3, respectively). These values, however, still allow for variations between centers. In addition, the possible use of different quantification platforms may contribute to variations, as different platforms may yield different results (A. C. Fluit, unpublished observations). Moreover, only 2 μl of the DNA solution is added, and small absolute variations lead to a large relative variation in volume. Furthermore, amplification products of different lengths are generated with one amplification protocol, leading to competition for amplification. The variations in DNA quality and amounts may be addressed by a more robust

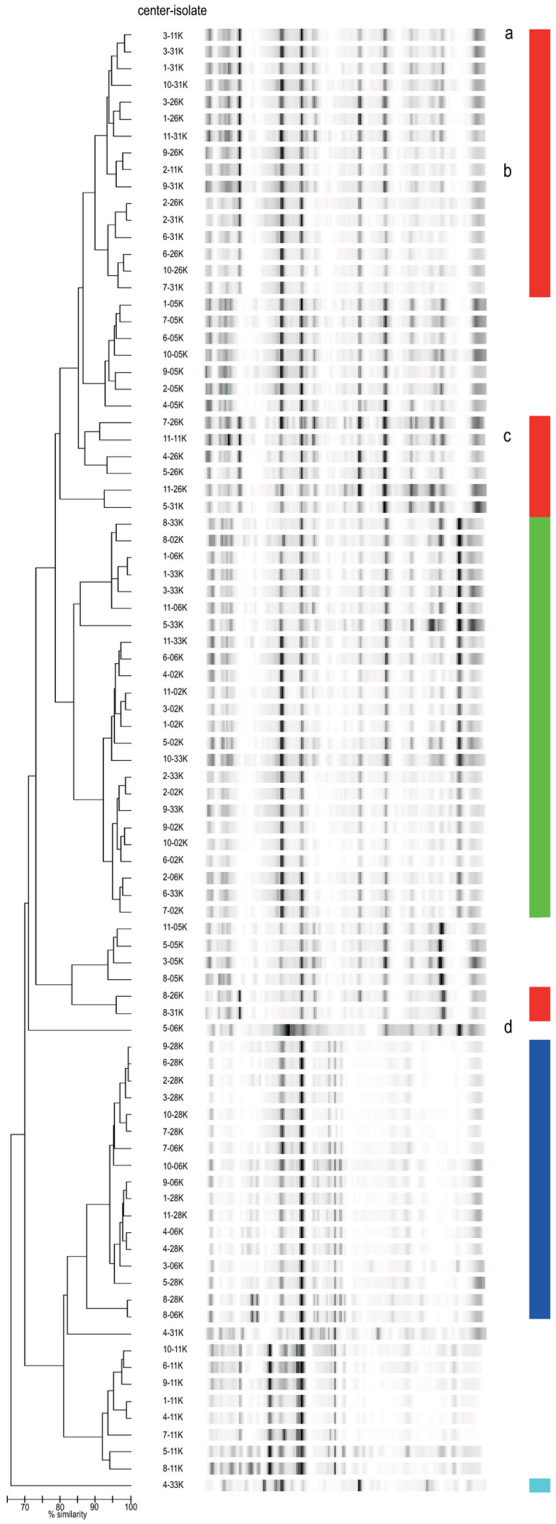

FIG 3 Clonal complex of isolates 02K, 05K, 06K, 11K, 26K, 28K, 31K, and 33K according to the central analysis. Three clusters were detected. The clusters correspond to the clusters and unique isolates in Fig. 2B and are indicated by colors. Red is equivalent to light green in Fig. 2B, green is equivalent to medium green, blue is equivalent to dark green, and light blue is equivalent to light blue; the other isolates have unique types. The isolates indicated by the a, b, and c labels are 11K isolates that did not group with the other 11K isolates (key 79 to 86). The pattern for the isolate labeled d is most likely due to a poor DNA sample.

protocol. Automatic DNA extraction can be useful for better control of the DNA amounts and quality. It should be noted, though, that the use of different types of automatic extraction machines still might contribute to problems with the reproducibility of amplification.

The final factors that contributed to the differences in the assignment of the isolates to different clusters were errors in administration, exchange of isolates and/or results, and failure to adhere to the protocol. These factors were most notable with the isolates included for analysis that generated poor signals or warning signals, even if a good result from a retest was present.

Despite the ability of the amplification protocol and the DL system to type every isolate in most centers, some centers reported isolates that were nontypeable according to the given criteria. Some centers retested isolates because the quality of the initial data was unacceptable. All centers performed at least a small number of retests, whereas a few of them required a considerable number of retests, although the numbers of retests varied greatly; e.g., one center retested 3 isolates once, and another center retested 90% (70 of 78) of the isolates and retested 1 isolate seven times. This also indicates that adequate training is required. No pattern with particular isolates being retested more often than others was discerned among the centers.

We conclude that the DiversiLab system has the potential for indicating the occurrence of outbreaks in an international setting, at least for *E. coli* and *Klebsiella* spp., although with a lower discriminatory power than that of PFGE. However, this will require more reproducible DNA amplification and isolation methods, strict adherence to protocols, and an international database to allow the comparison of isolates. In addition, reference isolates should be used with every chip to confirm the quality of each amplification.

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